Rock pool fish use a combination of colour change and substrate choice to improve camouflage

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Abstract

Camouflage can be achieved by both morphological (e.g. colour, brightness and pattern change) and behavioural (e.g. substrate preference) means. Much of the research on behavioural background matching has been conducted on species with fixed coloration and body patterns, while less is known about the role background choice plays in species capable of rapid (within minutes or seconds) colour change. One candidate species is the rock goby (*Gobius paganellus*), a common rock pool fish capable of rapid changes in colour and brightness when placed on different backgrounds. However, their ability to match different backgrounds is not unbounded, with some colours and brightness being easier to match than others thus raising the possibility that gobies may use behavioural background matching to make up for their limited ability to match certain backgrounds. We used digital image analysis and a model of predator vision to investigate the ability of rock gobies to match chromatic (beige and greenish-grey) and achromatic (varying brightness) backgrounds. We then conducted choice experiments to determine if gobies exhibited a behavioural preference for the backgrounds they were best at matching. Gobies rapidly changed their colour and brightness when placed on the different backgrounds. However, the level of camouflage differed between backgrounds, whereby fish were better at matching beige than greenish-grey, and darker over lighter backgrounds. When given the choice gobies displayed a behavioural preference for the backgrounds they were best at matching. Our findings therefore show that rock gobies, and likely other animals, use a combination of morphological and behavioural means to achieve camouflage and in doing so mitigate limitations in either approach alone.

Keywords: background matching, behavioural background matching, camouflage, colour change, fish

Introduction

Camouflage through cryptic coloration is one of the most widespread anti-predator strategies in nature (Cott, 1940; Ruxton, Sherratt, & Speed, 2004; Stevens & Merilaita, 2009; Thayer, 1909). The term crypsis is used to describe coloration that primarily prevents detection, and encompasses several different forms of camouflage including countershading, background matching, and disruptive coloration (Stevens & Merilaita, 2009). Probably the most common form of crypsis is background matching (Merilaita & Stevens, 2011), which occurs when an animal's appearance matches the overall colour (hue and saturation), brightness, and pattern of one or several background types (Stevens & Merilaita, 2009).

The overall appearance of many species, for example numerous members of the Lepidoptera, has evolved to match the appearance of specific backgrounds, such as tree bark, (e.g. Kettlewell, 1955; Endler, 1984). Other species may evolve coloration and body patterns that are a compromise between the attributes of multiple backgrounds rather than specialising to match a single specific background (Houston, Stevens, & Cuthill, 2007; Merilaita, Lyytinen, & Mappes, 2001; Merilaita, Tuomi, & Jormalainen, 1999). For crypsis to be effective, many animals of fixed appearance exhibit behavioural background matching, whereby they actively choose backgrounds that match their own species-, morph-, or individual-level appearance (e.g. Kettlewell & Conn, 1977; Kang et al., 2012, 2013; Lovell et al., 2013; Marshall, Philpot, & Stevens, 2016; Stevens et al., 2017; reviewed by Stevens & Ruxton, 2018). However, although a fixed camouflage pattern increases survival against predators (Troscianko, Wilson-Aggarwal, Stevens, & Spottiswoode, 2016) it can carry a number of costs (Ruxton et al., 2004). For instance, a fixed appearance restricts an animal to remain on a specific background and may prevent prey from taking advantage of potential opportunities, such as foraging on a non-matching substrate (Ruxton et al., 2004), and being limited in their ability

to cope with spatial or temporal uncertainty in the environment (Caro, Sherratt, & Stevens, 2016).

One way that animals may overcome the constraints that arise due to camouflage being tied to a specific background type(s) is to actively alter their appearance in response to changes in their visual background (Duarte, Flores, & Stevens, 2017; Stuart-Fox & Moussalli, 2009). Colour change (here used to encompass changes in pattern and brightness as well as colour) has been documented in many animal linages, including reptiles (e.g. Stuart-Fox, Moussalli, & Whiting, 2008), fish and amphibians (e.g. Sköld, Aspengren, & Wallin, 2013), crustaceans (e.g. Stevens, Rong, & Todd, 2013; Stevens, Lown, & Wood, 2014b), and cephalopods (e.g. Hanlon & Messenger, 1988). While cephalopods provide the most extensively studied examples of rapid (seconds) colour change it is also common among teleost fishes (Sköld et al., 2013), with several species known to change colour and brightness in response to changes in the prevailing light conditions of their environment (e.g. Clarke & Schluter, 2011; Kelley et al., 2012). Other species change colour and brightness to match that of different substrates (Kelman, Tiptus, & Osorio, 2006; Lanzing, 1977; Ramachandran et al., 1996; Sumner, 1935). The speed of colour change does, however, vary considerably between species. Among flatfish for instance, species such as English sole (*Parophrys vetulus*), northern rock sole (*Lepidopsetta polyxystra*), and Pacific halibut (*Hippoglossus stenolepis*) take several hours to days to fully change colour (Ryer, Lemke, Boersma, & Levas, 2008), while eyed flounder (*Bothus ocellatus*) take 2-8 seconds to match their background (Ramachandran et al., 1996).

While the ability to change colour and brightness for camouflage likely provides a clear survival advantage (Duarte et al., 2017; Fairchild & Howell, 2004; Sumner, 1935), the ability of animals to match different backgrounds is not unbounded, with some backgrounds being

92 easier to match than others (e.g. Stevens, Lown, & Denton, 2014a). Furthermore, colour
93 change is also widely thought to involve some degree of energetic cost and constraints, likely
94 limiting its use (Rodgers et al., 2013; Polo-Cavia & Gomez-Mestre, 2017; reviewed by
95 Duarte et al., 2017). Potentially as a result of these and other issues, a number of colour
96 changing species also exhibit some degree of behavioural background matching (e.g. Garcia
97 & Sih, 2003; Ryer et al., 2008; Tyrie et al., 2015; Duarte et al., 2017; Polo-Cavia & Gomez-
98 Mestre, 2017; Stevens & Ruxton, 2018). The peacock flounder (*Bothus lunatus*), for
99 example, prefers substrates that it is able to match while avoiding those it cannot (Tyrie et al.,
100 2015). However, the relative importance of both colour and brightness change and substrate
101 choice for camouflage is still little known, and rarely quantified in the context of predator
102 vision.
103
104 Stevens et al. (2014a) found that although rock gobies (*Gobius paganellus*) are capable of
105 rapid (occurring within one minute) changes in colour (hue and saturation) and brightness,
106 the level of achievable background matching depended heavily on the colour and brightness
107 of their background. However, the coloured backgrounds used by Stevens et al. (2014a) did
108 not resemble those found within natural habitats. Smithers et al (2017) went on to show that
109 these fish also change their body pattern when placed on backgrounds with different sized
110 features. When the fish were tested on backgrounds resembling different background marking
111 sizes found in natural substrates, the level of camouflage achievable differed greatly between
112 backgrounds (Smithers et al., 2017). This raises questions regarding whether or not fish such
113 as the rock goby also exhibit behavioural background matching, to make up for their limited
114 ability to match certain backgrounds.
115

This study aims to test whether intertidal species such as the rock goby use background choice, in combination with colour and brightness change to achieve camouflage. Being an intertidal species, rock gobies are exposed to both marine and terrestrial predators and a wide range of backgrounds and physical disturbance such as tides and waves that can push individuals around the habitat. We first investigated the ability of rock gobies to match i) two different hues (beige and greenish-grey) inspired by natural substrates found within rock pools in experiment 1, and ii) four achromatic backgrounds that differ in brightness (black, dark grey, light grey, and white) in experiment 2. In the rock pool environment where the work was undertaken, there exists a range of features (including rocks) that vary from bright white through to black. We then tested whether the fish displayed a behavioural preference for either i) beige or greenish-grey in experiment 3, and ii) black or white in experiment 4. We predicted that the fish would be better at matching one hue (in experiment 1) or brightness (in experiment 2) over the others tested, and that fish would display a behavioural preference for the hue (in experiment 3) or brightness (in experiment 4) that they were best at matching. If, however, there was no difference in the level of background matching camouflage between the backgrounds tested then we predict the fish to show no behavioural background preference. Digital image analysis and a model of predator vision were used to quantify changes in hue, saturation, luminance (perceived brightness), and overall camouflage as per previously outlined methods.

Methods

The study was carried out *in situ* on Gyllyngvase beach, Falmouth, Cornwall, UK (50.1441° N, 5.0684° W) where rock gobies were collected using a dip net from the local rock pools.

Animal welfare note

All work was conducted under approval from the University of Exeter Biosciences ethics committee (application 2015/739). Gyllyngvase beach is public land and no further licences or permits were needed. The experimental setup was designed to minimise stress to the animals and all individuals were returned unharmed to their original rock pool area immediately after being tested. Rock gobies are not an endangered or protected species.

Generating the experimental backgrounds

All backgrounds were generated in the graphics program inkscape v0.48 and printed on either HP LaserJet Tough paper (Hewlett Packard, Palo Alto, USA) (experiment 1) or Xerox Premium NeverTear waterproof paper (Xerox, CT, USA) (all other experiments) with a Hewlett Packard LaserJet 500 color M551 PCL6 printer.

<u>Beige and green-grey chromatic backgrounds</u>

Our approach to generating the printed chromatic backgrounds was similar to that used by Kang, et al. (2016). To make our printed colours somewhat representative of those within the rock pools we took photos (taken from above) of two common substrate types that we subjectively classified as being either beige (four photos of wet sand) or greenish-grey (nine photos of rock which was often covered in a greenish biofilm) (see supplementary figure A1 for examples). A Spectralon grey reflectance standard (Labsphere, Congleton, UK), which reflects 40% of all wavelengths between 300 and 750 nm was included in each photo (see

section below on image analysis for details on camera set up). Since it is not possible to print in ultraviolet (UV), photos were only taken in human visible light and not UV light. However, this should not be an issue since colour vision in gobies, which are potential trichromats, probably lacks UV sensitivity (Utne-palm & Bowmaker, 2006) and so colour and luminance change or background choice should not be affected by missing this part of the spectrum.

Conventionally, reflectance is measured using a spectrometer, but here we measured reflectance based on each of the camera's RGB colour channels (longwave (LW), mediumwave (MW) and shortwave (SW)), whereby a value of 65535 on a 16-bit scale is equal to 100% reflectance (Stevens, Párraga, Cuthill, Partridge, & Troscianko, 2007; Troscianko & Stevens, 2015). The appearance of printed colours is dependent not just on the pixel RGB values but also on the properties of the printer. It was therefore necessary to calibrate the colours before the final backgrounds could be printed (Cuthill, Stevens, Sheppard, & Maddocks, 2005; Kang et al., 2016). To do this we used the mean RGB values for the two natural background colours to generate several grids of similar colours of different brightness using the RGB scales in inkscape. These grids were then printed and photographed under an Iwasaki eyeColour MT70D E27 6500K arc lamp. We then chose the colours that had the most similar RGB values to the mean RGB values of the natural backgrounds. To match the brightness of the beige and greenish-grey backgrounds (hereafter referred to as BE and GG respectively) we created a new grid for each of the two colours in which we manipulated the brightness, while maintaining the hue, by changing the RGB values proportionately. Using the value from the camera's green channel as our measure of brightness in accordance with previous work (Smithers et al., 2017; Spottiswoode & Stevens, 2011; Stevens et al., 2013) we chose the two colours that that had a reflectance value of 40%

± 2% in the camera green channel. Note that BE and GG were designed to be broadly representative of the natural colours found in rock pools and were not designed to be exact replicas of the natural substrates they were inspired by. A grey colour (R=G=B) of the same brightness (based on the camera's green channel) as the experimental backgrounds was used as the starting background for the chromatic experiments.

Achromatic backgrounds

For the achromatic experiments four backgrounds of different brightness (black, dark grey, light grey, and white, hereafter referred to as BK, DG, LG, and WH respectively) were chosen from a grid of grey squares (RGB values ranging from 0:0:0 to 255:255:255) using the method described above. We used the darkest grey (R=G=B=0) for BK and the plain paper (R=G=B=255) for WH. DG and LG had a relative reflectance of 25% and 75% respectively to BK and WH (based on the camera's green channel). A grey with a reflectance midway between the BK and WH, and DG and LG was used as the starting background for the achromatic experiments.

Experimental set up

Experiments were conducted in a 400 x 300 x 65 mm plastic tray using similar methods to Smithers et al. (2017). The tray was divided into four 185 x 130 mm sections in experiment 1, eight 85 x 130 mm sections in experiment 2, and two 370 x 130 mm sections in experiments 3 and 4 using vertical acrylic walls that were either fixed in place using aquarium safe silicone adhesive or held between transparent slide binders glued to the walls enabling these dividers to be removed to facilitate the movement of fish between sections. For experiment 1 the bottom and sides of the four sections were covered by either BE or GG (supplementary figure A2a). A separate 360 x 250 x 50 mm starting tray was covered in the starting grey for

experiment 1. For experiment 2 the four middle compartments were covered with the intermediate starting grey, while the four outside compartments were covered with either BK, DG, LG or WH (supplementary figure A2b). For experiments 3 and 4 half of each section was covered in either BE or GG, or BK or WH respectively (supplementary figures A3 and A4). In the middle of the two sections were two sliding dividers set at a 45° angle to the bottom of the tray and a 90° angle from each other. The dividers for experiments 3 and 4 were covered with the grey starting background used in experiments 1 and 2 respectively and when in place these dividers formed a small compartment in which fish were placed before starting the experiment. Fresh seawater, filled to a depth of approximately 20 mm, was used for each fish.

Experimental procedure

Colour and luminance change (experiments 1 and 2)

The general protocol was similar to that used by Smithers et al. (2017). A total of 20 fish were used in experiment 1 and 80 fish in experiment 2 (20 fish per background). Fish were tested in size matched blocks (to within ~20 mm) in which individuals were tested simultaneously ± 15 min. Blocks consisted of two fish in experiment 1 and four fish in experiment 2.

For experiment 1, each fish was tested on both backgrounds. The first background that fish were placed on was alternated so that half were tested on GG first and half on BE first. Fish were acclimatised on the starting grey for a minimum of 15 min in order to remove some individual differences in colour between fish prior to starting the experiment. Following this each fish was photographed (0 min) in both visible and UV light before being transferred to the first experimental background. Fish were photographed at approximately 1, 3, 5, 10, and

30 min. After being tested for 30 min on the first background each fish was photographed again (to be used as 0 min for the second background) and then moved to the second background where it was photograph as before. Experiment 2 followed the same procedure except each fish was only tested on a single background and photographs were taken at 1, 5, and 30 min.

Photography and image analysis followed previous studies (e.g. Stevens et al., 2014a; Smithers et al., 2017). All photographs were taken using a Nikon D7000 digital camera, which had undergone a quartz conversion to enable photos to be taken in both visible and UV light (Advanced Camera Services, Norfolk, UK) and fitted with a Nikon 105 mm Nikkor lens. All photos were taken in RAW format with manual white balance and fixed aperture and ISO (the sensitivity of the camera's sensor to light) settings using manual focus. The human visible photos were taken using a UV/infrared (IR) blocking filter which transmits wavelengths of 400-700 nm (Baader UV/IR Cut/L Filter) and UV photos taken using a UV pass and IR blocking filter which transmits wavelengths between 300 and 400 nm (Baader U filter). A custom made filter slider was used to quickly move between the two filters. A black and white reflectance standard (made from 10 x 10 mm sections of zenith diffuse sintered PTFE sheet, Labsphere), with a scale bar, was included in all photos taken to account for difference in lighting conditions at different times, and on different days. Photos were taken from above using a tripod and a photographic umbrella (Neewer, Guangdong, China) shaded the trays from direct sunlight.

Image analysis

Image analysis was conducted using the 'Multispectral Image Calibration and Analysis Toolbox' (Troscianko & Stevens, 2015) in accordance with previous studies (e.g. Stevens et

al., 2014a; Marshall et al., 2016; Smithers et al., 2017). Briefly, the visible and UV photos were combined into a single multispectral image. The area of the fish's body was selected (as was a sample of the background) as a region of interest and measured to acquire values for photon catch. Further details about this process are provided in the supplementary material. This study analysed changes in colour and luminance as perceived by shore birds, a potential predator of rock pool fish during low tide, in accordance with previous work (e.g. Stevens et al., 2014a,b; Smithers et al., 2017). We used spectral sensitivity data from the peafowl (*Pavo cristatus*) (Hart, 2002) under a D65 standard irradiance spectrum to convert from camera to avian colour space using a polynomial mapping technique (Stevens et al., 2007; Troscianko & Stevens, 2015). The peafowl is regularly used to model vision in species, such as the majority of shorebirds, which have a 'violet' sensitive (V) visual system (Ödeen, Håstad, & Alström, 2009). One exception to this are gulls which differ from shore birds in that they have a UV visual system (Ödeen et al., 2009). However, the differences in the perception between these two systems will be small since both the backgrounds and the fish had relatively low levels of UV reflectance. Compared to modelling predicted cone catch values with reflectance spectra, this mapping technique is highly accurate, with very low levels of potential error and R^2 values for each channel from 0.96 to 0.98 between derived cone catch values based on spectrometry and cameras (Pike, 2011; Stevens & Cuthill, 2006; Troscianko & Stevens, 2015).

Two metrics of 'colour' were calculated. First, saturation (the amount of a given colour compared to white light) was defined as the distance of an object from the achromatic grey point in a tetrahedral colour space (Endler & Mielke, 2005; Stevens, Lown, & Denton, 2014). Values of saturation are on a scale of 0 to 0.75 whereby the higher the value the more saturated the colour. Hue was used as a measure of colour type in accordance with previous

studies (e.g. Stoddard & Prum, 2008; Spottiswoode & Stevens, 2011). Our approach was broadly based on the way that opponent colour channels in animal vision are thought to work. Unfortunately, the opponent channels that exist in birds are not fully known and so cannot be modelled directly to obtain a measure of hue. Therefore, we simply aimed to define hue in a manner based on colour channels, calculated in the form of an intuitive ratio (Komdeur, Oorebeek, Overveld, & Cuthill, 2005), but we do not attempt to model real opponent channels. For experiment 1 we followed the approach set out in previous studies that used a principal component analysis to extract the main axis of variation that exists in colour space, and in turn use this to determine the most logical colour channel(s) (Spottiswoode & Stevens, 2011; Stevens, Lown, & Wood, 2014). A PCA was performed on a covariance matrix of the standardised values for the four colour channels and the resulting principal components (PCs) were used to determine the most logical opponent model for calculating hue (conducted in IBM SPSS Statistics v21). PC1 explained 83% of the variance and was equivalent to the following colour channel: hue = $(\frac{UV+SW+MW}{3})/LW$. The lower the value of hue the more LW, or 'redder', the fish conceptually appears to avian vision. Since only achromatic backgrounds were used in experiment 2 we used a different approach to calculating hue in accordance with previous work (Stevens, Lown, & Denton, 2014) whereby hue was defined as ((LW+MW)– (SW+ UV))/(LW+MW+SW+UV). The cone catch values for the double cones were used as a measure of luminance (perceived brightness) in accordance with literature suggesting that these receptors underpin achromatic perception in birds (Jones & Osorio, 2004; Osorio, Miklósi, & Gonda, 1999; Osorio & Vorobyev, 2005; Osorio, Vorobyev, & Jones, 1999). Luminance is on a scale of 0 to 1 with brighter objects resulting in higher values.

For experiment 1, to determine the level of background matching for each fish when viewed against its background by avian predators, we used a log form of the tetrachromatic

version of the Vorobyev-Osorio colour discrimination model (Vorobyev & Osorio, 1998). An assumption of this model is that visual discrimination is limited by receptor noise (Vorobyev & Osorio, 1998). The model uses differences in colour based on photon catch values and includes estimates of neural noise and relative photoreceptor properties. A Weber fraction value of 0.05 was used for the most abundant cone types in accordance with previous work (e.g. Eaton, 2005; Endler & Mielke, 2005) and the relative proportions of the different cone types in the retina of the peafowl (LW = 0.95, MW = 1, SW = 0.86, UV = 0.45) (Hart, 2002). The model outputs 'just noticeable differences' (JNDs), whereby a value of less than 1 means that two stimuli are likely indiscriminable from one another, and increasing values above this mean that they are increasingly likely to be distinguishable (Siddiqi, Cronin, Loew, Vorobyev, & Summers, 2004). For experiment 2 we used an achromatic analysis based on Siddiqi et al. (2004), where comparisons are based on luminance differences obtained from the double cones. When generating JNDs, fish at 0 min were compared to the sample of the background at 1 min.

Background choice experiments (experiments 3 and 4)

The aim of the choice tests was to determine whether fish displayed a behavioural preference for BE or GG (experiment 3), or BK or WH (experiment 4), and to determine whether or not the background the fish has been acclimatised to affected their preference. A total of 40 fish were used for each experiment (20 per background). Prior to the experiment, fish were randomly assigned to one of the two backgrounds in size-matched pairs and given a minimum of 30 min to acclimatise in a separate tray. Next each fish was placed in the small compartment created by the two starting grey dividers for a few seconds before starting the experiment. The experiment started as soon as the dividers were removed. If a fish was half way between both backgrounds or made no obvious choice after removing the dividers we

waited until the fish had completely moved onto one of the backgrounds (i.e. 100% of the fish's body was on one colour) before starting the trial. Hereafter, a fish was said to have changed to a different background if at least 50% of its body including its head was on that background. Trials lasted for 10 min and each pair was tested almost simultaneously. Trials were recorded from above using a Sony HDR-PJ810 Handycam. The tray was filled to a depth of 30 mm with sea water that was changed after each trial. Each video was scored post-hoc using windows media player. For each fish we determined the amount of time spent on each background.

Statistical analysis

Colour and luminance change experiments

To analyse the data from experiments 1 and 2 we used general linear mixed effects models in the lme4 package in R (Bates, Maechler, Bolker, & Walker, 2014). The response variables were luminance, hue, saturation, and JNDs. These were natural log transformed to correct for positive skew in the distribution of the residuals. For experiment 2, luminance JNDs did not require transformation. In all models, test background, time point, and an interaction between the two, were included as categorical fixed effects, and fish identification was included as a random effect factor. The latter accounts for non-independence between time points (and backgrounds in experiment 1) and prevents pseudoreplication. For experiment 1, order of testing (i.e. whether the background was the first or second the fish was tested on) was included as an additional fixed effect. Models were fitted by maximum likelihood and compared with one another using a likelihood ratio test (LRT) to sequentially remove non-significant interactions and effects. Initial analysis revealed that all statistically significant changes in luminance, colour variables, and camouflage occurred within 1 min. For

simplicity we therefore reanalysed the results using only the data for 0 and 1 min. For graphs showing the data for all of the time points tested see supplementary figures A5 and A6.

Choice experiments

For each individual fish we randomly assigned one of the two backgrounds and performed a beta regression using the R package 'betareg' (Cribari-Neto & Zeileis, 2010), whereby the response variable was the proportion of time the fish spent on their randomly assigned background. We included the colour of the randomly chosen background and the colour of the acclimation background as fixed effects along with an interaction between the two. In this way we tested whether the fish preferred, i) one background over the other; ii) the background they were acclimated on; or iii) whether background preference depended on the acclimation background. Beta regression requires that all observations are between 0 and 1 (i.e. no proportion can be exactly 0 or 1). Because some of the fish did spend all of their time on a single background (thus generating a proportion of exactly 0 or 1) we transformed all values to a variable in the open (0,1) interval by taking the weighted average: transformed proportion = $[y(N-1)+0.5]/N$, where y is the proportion of time the fish spent on their randomly assigned background, and N is the sample size (which was 20 for each treatment) (Cribari-Neto & Zeileis, 2010; Smithson & Verkuilen, 2006). We used a LRT to compare models and sequentially remove non-significant terms. Only significant interactions are reported in the results. All statistical analysis and graphical modelling was carried out in R (R Core Team, 2017).

Results

Colour and luminance change experiments

Experiment 1

Luminance

The fish showed no difference in luminance between BE and GG (LRT: background: χ^2_1=0.83, *P*=0.36; Figure 1a), but luminance on both backgrounds increased after 1 min (time: χ^2_1=17.77, *P*<0.001). There was also a significant effect of order; i.e. whether it was the first or second background the fish were tested on, (order: χ^2_1=18.547, *P*<0.001) whereby fish tested on GG second tended to have a slightly higher luminance than those tested on GG first.

Hue

There was no difference in hue between fish on BE and GG at the start of the experiment (compare BE1 vs GG1 at 0 min in figure 1b), but at 1 min fish on both backgrounds showed a decrease in hue value. Specifically, fish on BE were more LW in hue than those on GG (background-time interaction: χ^2_1=24.37, *P*<0.001; Figure 1b). To better understand this change in hue we plotted the data in tetrahedral colour space (figure 2). While fish on both backgrounds turned more LW in colour compared to their colour on the starting grey, figure 2 shows that fish on GG also turned more MW than fish on BE. This is consistent with the greener hue of this background. The order of testing had no effect on the final hue of the fish at 1 min (compare GG1 vs GG2, and BE1 vs BE2 at 1 min in Figure 1b) but there was an effect of order a 0 min (compare GG1 vs GG2 at 0 min) because the fish were moved from their first background straight to their second background (compare GG2 at 0 min vs BE1 at 1 min) (order-time interaction: χ^2_1=19.09, *P*<0.001).

Saturation

As with hue, there was no difference in saturation between fish at the start of the experiment (compare BE1 vs GG1 at 0 min in figure 1c) but fish on both backgrounds become more saturated after 1 min. Specifically, fish on BE became more saturated than those on GG (background-time interaction: χ^2_1=15.56, P<0.001; Figure 1c). This change in saturation can also be seen in figure 2 in which the position of the fish in tetrahedral colour space moves further from the achromatic centre after 1 min. As was the case for hue, the order of testing resulted in a difference in saturation at 0 min particularly between fish on GG1 vs GG2 (order-time interaction: χ^2_1=17.31, P<0.001).

Colour JNDs

There was no difference in the level of background matching camouflage at 0 min (compare BE1 vs GG1 at 0 min in figure 1d) but fish on BE showed a significant improvement in background matching after 1 min (indicated by a decrease in JNDs) while fish on GG showed a slight decrease in their level of camouflage (background-time interaction: χ^2_1=6.89, P=0.009; Figure 1d). This difference in background matching between the two backgrounds is apparent in figure 2, which shows fish on BE are closer in colour to their background at 1 min than fish on GG. There was no effect of order of testing (order: χ^2_1=2.62, P=0.16).

Experiment 2

Luminance

There was a significant change in luminance after 1 min whereby fish on WH and LG increased their luminance while those on BK and DG, become darker (background-time interaction: χ^2_3=121.56, P<0.001; Figure 3a).

Hue

The greatest change in hue was for fish on BK which become more SW and UV after 1 min. Fish on DG and LG showed less change, while those on WH appeared more LW and MW at 1 min (background-time interaction: χ^2_3=32.61, P<0.001; Figure 3b). When plotted in tetrahedral colour space it is apparent that fish on DG and BK are more variable in colour at 1 min than fish on LG or WH (figure 4).

Saturation

Fish on WH showed the greatest change in saturation after 1 min and were more saturated than fish on the other three backgrounds at 1 min (background-time interaction: χ^2_3=14.56, P=0.002; Figure 3c). There was no change in the saturation for fish on LG while fish on the two darker backgrounds showed a small decrease in saturation at 1 min.

Luminance JNDs

There was a significant difference in the level of background matching camouflage between the four backgrounds, with fish being more camouflaged on the darker backgrounds. Fish on all backgrounds except DG showed an improvement in their level of background matching after 1 min with the greatest improvement shown by fish on WH and LG (background-time interaction: χ^2_3=35.01, P<0.001; Figure 3d).

Background choice experiments

Experiment 3

The gobies exhibited a significant preference for BE (χ^2_1=4.38, P=0.036; Figure 5a). This preference was strongest among fish acclimatised to BE, although acclimation background

was not found to have a significant effect on preference (χ^2_1=3.57, *P*=0.059). On average fish acclimatised on BE spent approximately 65% of their time on BE while those acclimatised on GG spent around 56% of their time on BE.

Experiment 4

All fish exhibited a very strong preference for BK (χ^2_1=31.06, *P*<0.001; figure 5b) regardless of the background they were acclimated on (χ^2_1=0.02, *P*=0.89). The mean percentage of time spent on the BK was approximately 84% for fish acclimatised on BK and approximately 79% for fish acclimatised on WH.

Discussion

We hypothesised that if there was a difference in the level of background matching camouflage between test backgrounds then rock gobies would exhibit a behavioural preference for the background they were best at matching. Our findings show this to be the case, demonstrating that, at least in the context of this study, rock gobies use behavioural choice to compensate for their limited ability to match certain backgrounds thus maximising their level of camouflage.

In experiment 1, rock gobies rapidly changed hue and saturation when placed on beige (BE) or greenish-grey (GG) backgrounds, but fish on BE showed the greatest change in colour and achieved a better background match. The fish also showed a small, but statistically significant, change in luminance that may be an unavoidable side effect of changing colour. When moved to a different background, fish improved their camouflage within 1 min showing that an individual's previous background has no effect on its ability to change colour to match a new one. In line with our hypothesis, fish exhibited a behavioural preference for

BE during the choice experiments. The finding that rock gobies were more camouflaged on BE (which broadly resembled the colour of sand) than on GG suggests there may be a greater selection pressure for fish to match sand-like colours compared to other colours such as GG. This may be due in part to the nature of the habitats in which these colours are most predominant. On the rocky shore where this study took place, green and grey colours are common within rock pools but less common outside this habitat. Since rock pools are extremely heterogeneous in their substrate composition there exists a range of different colours and selection to match any single colour may be small. In comparison, the beige colour of sand is subjectively one of the single most common substrates found in our study area, occurring in both the rock pools and nearby sandy shores. Habitats comprising mostly of sand tend to be more homogeneous and have fewer places for animals to take shelter, compared to rock pools that provide animals with numerous places to hide. Several flatfish species as well as cuttlefish are known to partially or completely bury in sand to increase crypsis (Ellis, Howell, & Hughes, 1997; Fairchild & Howell, 2004; Hanlon & Messenger, 1988; Ryer et al., 2008; Tyrie et al., 2015), but no such behaviour has been documented in rock gobies. Furthermore, selection pressure by visually hunting predators in heterogeneous environments such as rock pools may be lower than in homogeneous habitats dominated by sand since prey detection has been shown to be more difficult in complex habitats (Bond & Kamil, 2006; Dimitrova & Merilaita, 2012; Stoner & Titgen, 2003; Xiao & Cuthill, 2016). It is therefore plausible that there may be a higher predation risk on intertidal sandy shores for this species.

When placed on achromatic backgrounds, rock gobies rapidly changed their luminance resulting in an overall improvement in background matching camouflage that was most apparent on the lighter backgrounds. We also found a difference in the fish's hue and

507 saturation between backgrounds at 1 min, particularly between BK and WH. Any differences
508 in colour between fish and their background were, however, comparatively small relative to
509 differences in luminance, and so the perceptual effect on camouflage is likely to be relatively
510 small. The level of background matching was overall much better on the darker backgrounds
511 despite these fish showing the smallest change in luminance, similar to previous studies
512 (Stevens, Lown, & Denton, 2014). Very bright, purely achromatic, backgrounds are less
513 common within the rock gobies natural habitat, and so a limited ability to match our two
514 brightest backgrounds may not infer a considerable survival disadvantage for these animals.
515 This is particularly true given that the gobies displayed a strong avoidance of WH during the
516 choice experiments, choosing instead to spend the majority of their time on BK. This strong
517 preference for BK is in accordance with other studies which found that other species of fish,
518 as well as amphibians, also have a preference for dark backgrounds (Bradner & McRobert,
519 2001; Garcia & Sih, 2003; Kjernsmo & Merilaita, 2012). It is, however, in contrast to
520 behavioural choice in some other species whereby substrate choice can be individual-specific
521 (e.g. Lovell et al., 2013; Marshall et al., 2016; Stevens et al., 2017; Stevens & Ruxton, 2018).
522
523 It has been suggested that a preference for one substrate over another could be
524 detrimental to survival as predators may learn to search for prey on their preferred
525 background (Allen et al., 2010). However, a lack of background preference to reduce
526 predator learning needs to be balanced with a preference for substrates on which the prey is
527 able to camouflage best. The strength of a preference for a particular background exhibited
528 by any given species or individual may therefore be expected to depend on the ability of the
529 animal to change colour, luminance, and pattern to match different substrates. This is because
530 species with better dynamic background matching ability may evolve a weaker behavioural
531 preference compared to species with a more limited ability to change their appearance (e.g.

532 Allen et al., 2010; Tyrie et al., 2015). However, a preference for one background type may
533 not always infer an attempt at increasing crypsis. For instance, Polo-Cavia and Gomez-
534 Mestre (2017) found that, when in the presence of predator cues, larval newts increased the
535 amount of time they spent in light environments and suggested this was potentially the result
536 of an escape response to what the larvae likely perceived as shallower water, rather than an
537 attempt at improving crypsis. Moreover, habitat, and therefore background, preference is also
538 likely to depend on other factors such the availability of resources (e.g. Gilby et al., 2015).
539
540 In both experiments colour and luminance change occurred within 1 min. The speed
541 of change is important because it would allow gobies, and potentially other intertidal fish
542 with the ability to change their appearance, to move across multiple background types while
543 minimising the amount of time that the fish is contrasting against its background. It is
544 therefore not surprising that background preference was not influenced by acclimation
545 background in the choice experiments as the fish endure only a short-lived increase in their
546 conspicuousness while changing their appearance to match their preferred background. This
547 study augments the suggestion that rapid colour change functions to reduce predation risk in
548 heterogeneous habitats (Polo-Cavia & Gomez-Mestre, 2017; Smithers et al., 2017; Stevens,
549 Lown, & Denton, 2014).
550
551 Our findings show that while rock gobies were capable of rapid changes in colour and
552 luminance, certain backgrounds appeared easier to match than others thus gobies displayed a
553 behavioural preference for the backgrounds they were best at matching. Behavioural
554 background choice therefore appears to also play an important role in achieving camouflage
555 within this species in intertidal habitats. It is likely that other colour changing species will
556 also be better at matching certain backgrounds more than others, and like rock gobies they

may make up for these short falls in colour changing ability by exhibiting some degree of behavioural background matching. Overall, our study shows how a combination of behavioural substrate choice, and colour and luminance change is likely to be an important approach by which these and other animals can mitigate limitations in either approach alone to achieve camouflage. Work in future needs to separate out the relative importance and selection on background choice versus colour change in species with different life-histories.

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Appendix

Supplementary methods

Image analysis

Image analysis was conducted using the 'Multispectral Image Calibration and Analysis Toolbox' (Troscianko & Stevens, 2015). First, each image underwent linearization and standardization to control non-linear responses in image values that are produced by cameras in response to changes in light levels and illuminating conditions (Stevens et al., 2007; Troscianko & Stevens, 2015). Following this, the visible and UV photos from each time point were combined into a single multispectral image consisting of information from both the visible and UV channels. For each multispectral image, the area of the fish's body (not including the gills, eyes, or pectoral and caudal fins) was selected by hand and saved as a 'region of interest' (ROI). A 1 cm2 sample of the background next to the fish was also selected and saved as a ROI on all images (expect those taken at 0 min when the fish was on a different background). We then measured the value of photon catch based on the spectral sensitivity of avian predators (as described in the main manuscript).

Calculating Saturation

Saturation (the amount of a given colour compared to white light) was defined as the distance of an object from the achromatic grey point in a tetrahedral colour space (Endler & Mielke, 2005; Stevens, Lown, & Denton, 2014). This involved standardising the values for the four colour channels to a proportion of their total, in order to remove absolute variation in brightness. Next, the values were converted to X, Y, and Z coordinates in a tetrahedral colour

space (equations in Endler and Mielke (2005)). The more saturated a given colour is, the larger the distance from the achromatic grey point at the centre of the tetrahedron (Endler & Mielke, 2005). Values of saturation are on a scale of 0 to 0.75 whereby the higher the value the more saturated the colour.

Figure legends

Figure 1: Changes in (a) luminance, (a) hue, (c) saturation, and (d) colour just noticeable differences (JNDs) for rock gobies placed on beige (BE) and greenish-grey (GG) coloured backgrounds in experiment 1 at the start (0 min) and 1 min. Graphs show medians plus inter-quartile range (IQR), whiskers are lowest and highest values that are within 1.5*IQR from the upper and lower quartiles, outliers are shown by dots. GG1 and BE1 = the first background the fish were tested on. GG2 and BE2 = the second background the fish were tested on. Total sample size = 20 (20 per background).

Figure 2: The colour of the fish and test backgrounds from experiment 1 represented in avian tetrahedral colour space. (a) Fish on greenish-grey (GG) at 0 min (GG0), 1 min (GG1), and the GG background (GGbg). (b) Fish on beige (BE) at 0 min (BE0), 1 min (BE1), and the BE background (BEbg). (c) Comparisons between fish on GG and BE at 1 min and the two backgrounds (GGbg and BEbg). (d) Same as (c) but viewed from above. Inserts show a zoomed-in view of each plot. For simplicity and to avoid confusion, data for fish on their second background is excluded from (a) and (b) but (c) and (d) show data for fish on their first and second background.

Figure 3: Changes in (a) luminance, (b) hue, (c) saturation, and (d) luminance just noticeable differences (JNDs) for rock gobies placed on the black (BK), dark grey (DG), light grey (LG), and white (WH) backgrounds in experiment 2 at the start (0 min) and 1 min. Graphs show medians plus inter-quartile range (IQR), whiskers are lowest and highest values that are within 1.5*IQR from the upper and lower quartiles, outliers are shown by dots. Total sample size = 80 (20 per background).

Figure 4: The colour of the fish and test backgrounds from experiment 2 represented in avian tetrahedral colour space. (a) Fish on black (BK1), dark grey (DG1), light grey (LG1), and white (WH1) at 1 min. (b) Same as A but viewed from above. (c) Comparisons between fish on BK and WH at 1 min and the BK and WH backgrounds (BKbg and WHbg). (d) Same as (c) but viewed from above. Inserts show a zoomed-in view of each plot.

Figure 5: Proportion of time (mean percentage ± SE) rock gobies spent on (a) greenish-grey (GG, top bar) and beige (BE, bottom bar), or (b) white (WH, top bar) and black (BK, bottom bar) during the 10 minute background choice trials after being acclimated to one of the test backgrounds for 30 min. Total sample size in each experiment = 40 (20 per acclimation background).

Figure A1: Photographs of the different coloured substrates found within the rock pools on Gyllyngvase beach, Falmouth, UK. Examples of (a-b) beige sand and (c-d) rock gobies (Gobius paganellus) pictured among greenish-grey rocks that inspired the beige and greenish-grey backgrounds used in the chromatic experiments (experiments 1 and 3). Photos (a) and

(b) were taken by Samuel Smithers and (c) and (d) were taken by, and used with permission from, Alice Lown.

Figure A2: Experimental tray used for (a) experiment 1, and (b) experiment 2. Note that the colours of the backgrounds shown here may differ slightly from the actual colours of the calibrated printed background used in the experiments.

Figure A3: Experimental tray used for choice tests in experiment 3. (a) Diagram showing the experimental backgrounds (same as those used in experiment 1) and the design of the tray used for experiment 3, and (b) final tray with the starting dividers on the left side removed. Note that the colours of the backgrounds displayed here may differ slightly from the actual colours used in the experiment.

Figure A4: Experimental tray used for choice tests in experiment 4. (a) Diagram showing the experimental backgrounds (same as the black and white used in experiment 2) and the design of the tray used for experiment 4, and (b) final tray with the starting dividers on the left side removed. Note that the brightness of the backgrounds displayed here may differ slightly from the actual backgrounds used in the experiment.

Figure A5: Changes in (a) luminance, (b) hue, (c) saturation, and (d) colour just noticeable differences (JNDs) for rock gobies placed on beige (BE) and greenish-grey (GG) coloured backgrounds in experiment 1 at all time points. Graphs show medians plus inter-quartile

range (IQR), whiskers are lowest and highest values that are within 1.5*IQR from the upper and lower quartiles, outliers are shown by dots.

Figure A6: Changes in (a) luminance, (b) hue, (c) saturation, and (d) luminance just noticeable differences (JNDs) for rock gobies placed on the black (BK), dark grey (DG), light grey (LG), and white (WH) backgrounds in experiment 2 at all time points. Graphs show medians plus inter-quartile range (IQR), whiskers are lowest and highest values that are within 1.5*IQR from the upper and lower quartiles, outliers are shown by dots.

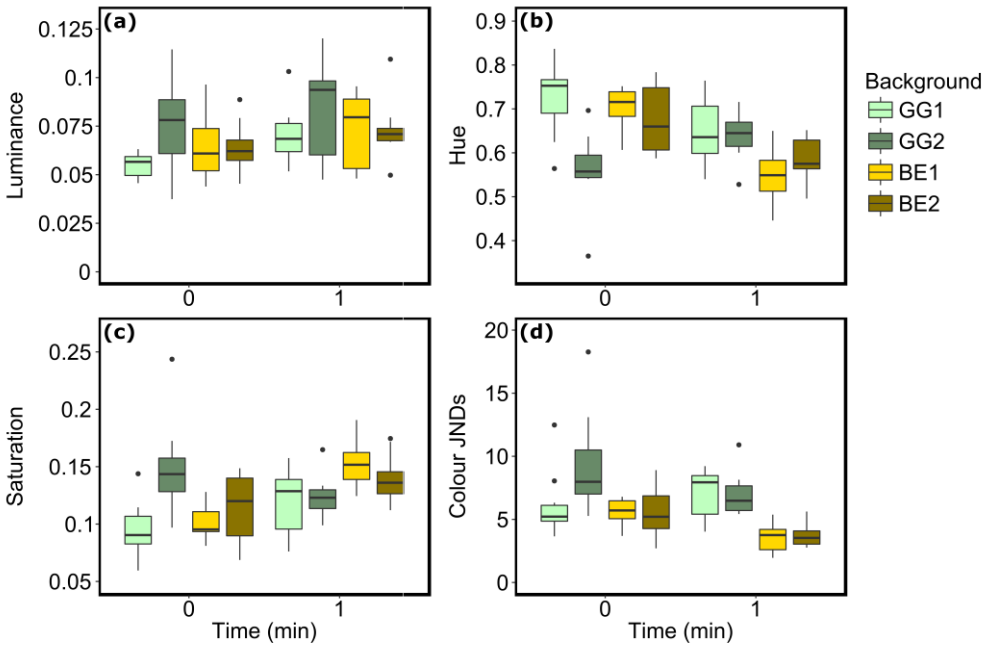


Figure 1

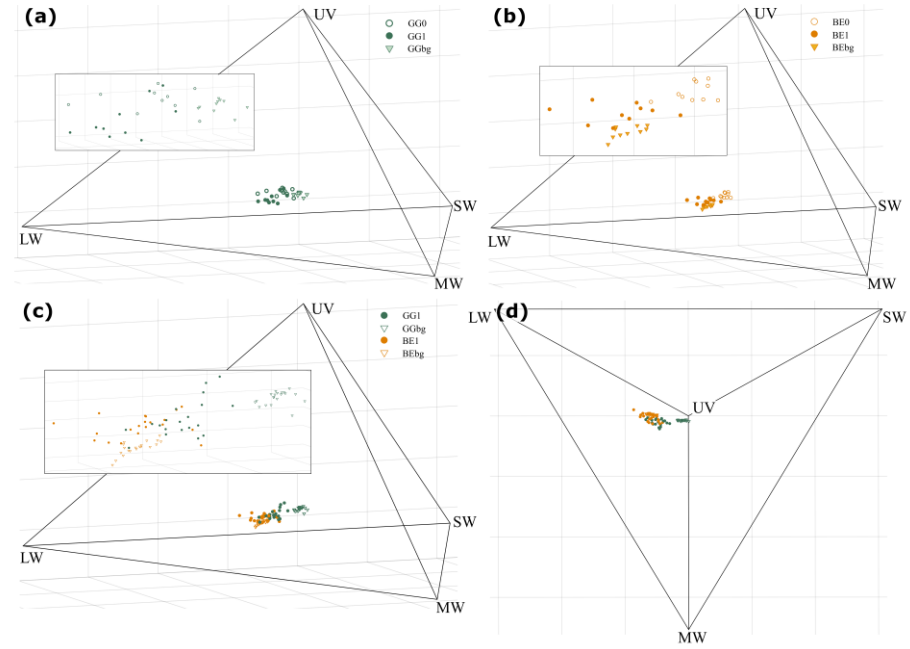


Figure 2

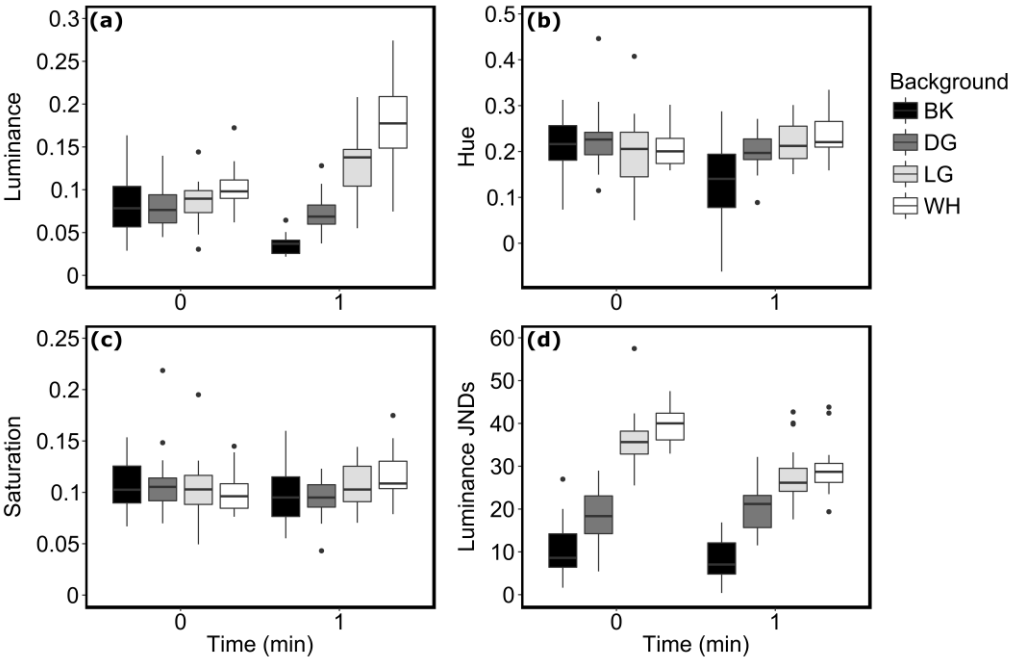


Figure 3

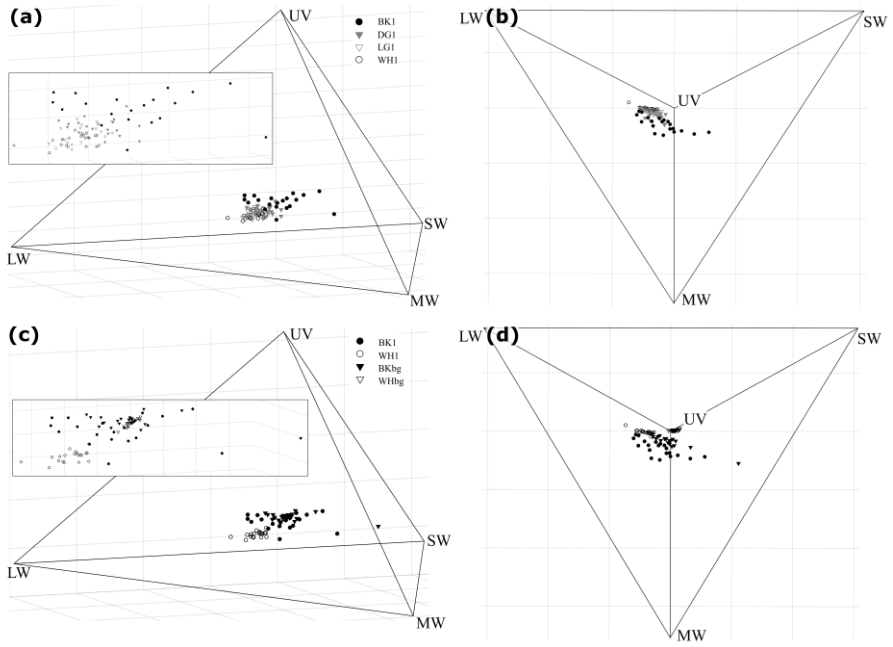

(a)
UV
BK1
DG1
LG1
WH1
SW
LW
MW
(b)
LW
SW
UV
MW
(c)
UV
BK1
WH1
BKbg
WHbg
SW
LW
MW
(d)
LW
SW
UV
MW

Figure 4

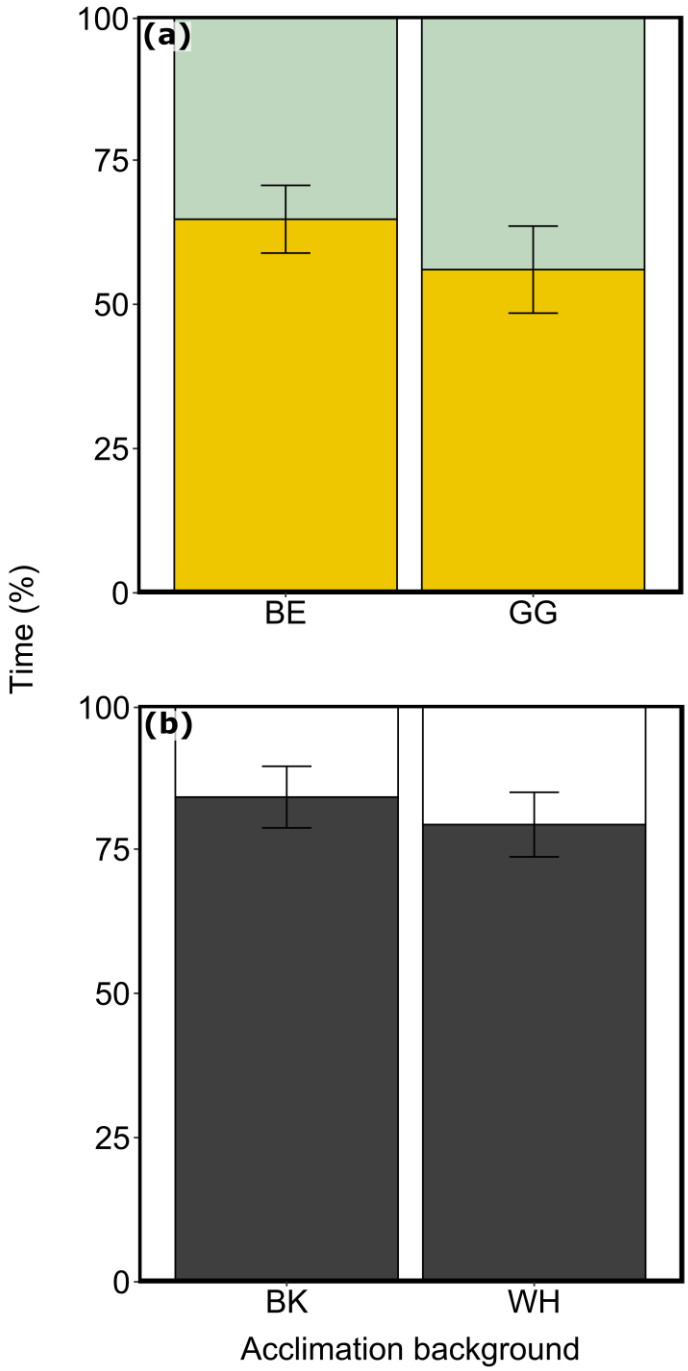
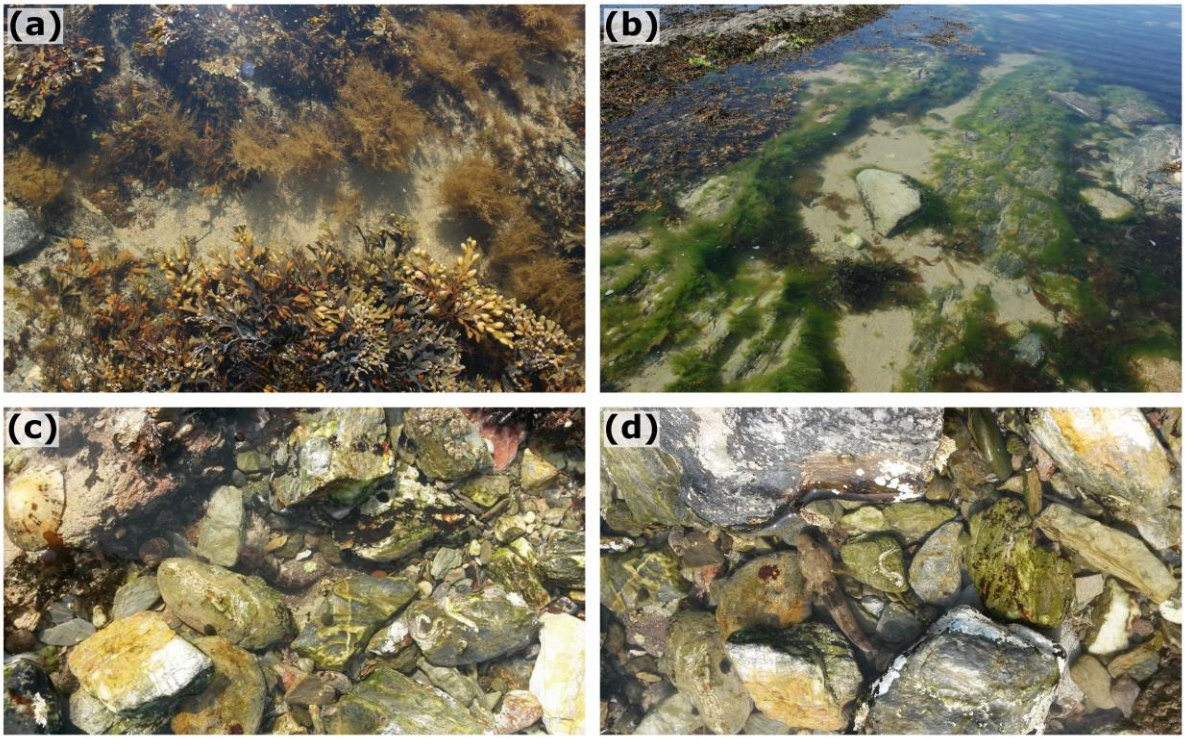
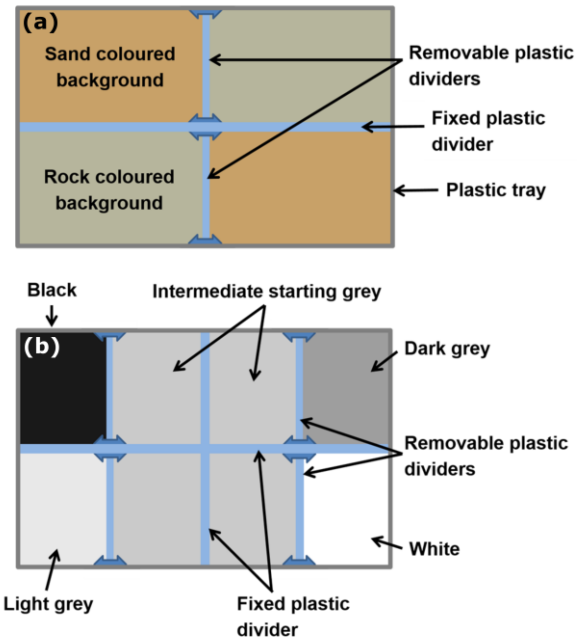

(a)
100
75
50
25
0
BE
GG
Time (%)
(b)
100
75
50
25
0
BK
WH
Acclimation background

Figure 5



907

908 **Figure A1**



909

910 **Figure A2**

911

912

913

914

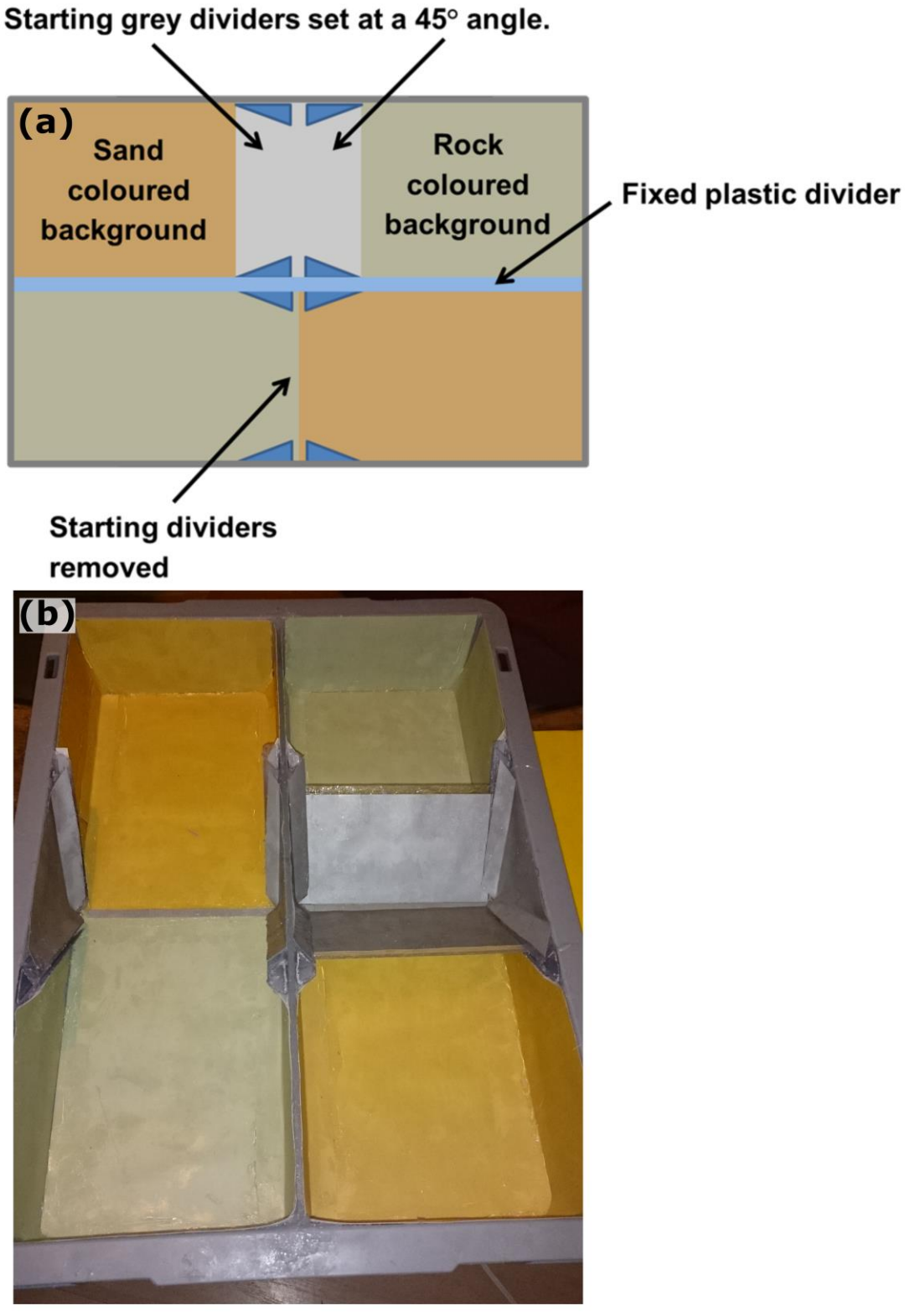

Starting grey dividers set at a 45° angle.
(a)
Sand coloured background
Rock coloured background
Fixed plastic divider
Starting dividers removed
(b)

Figure A3

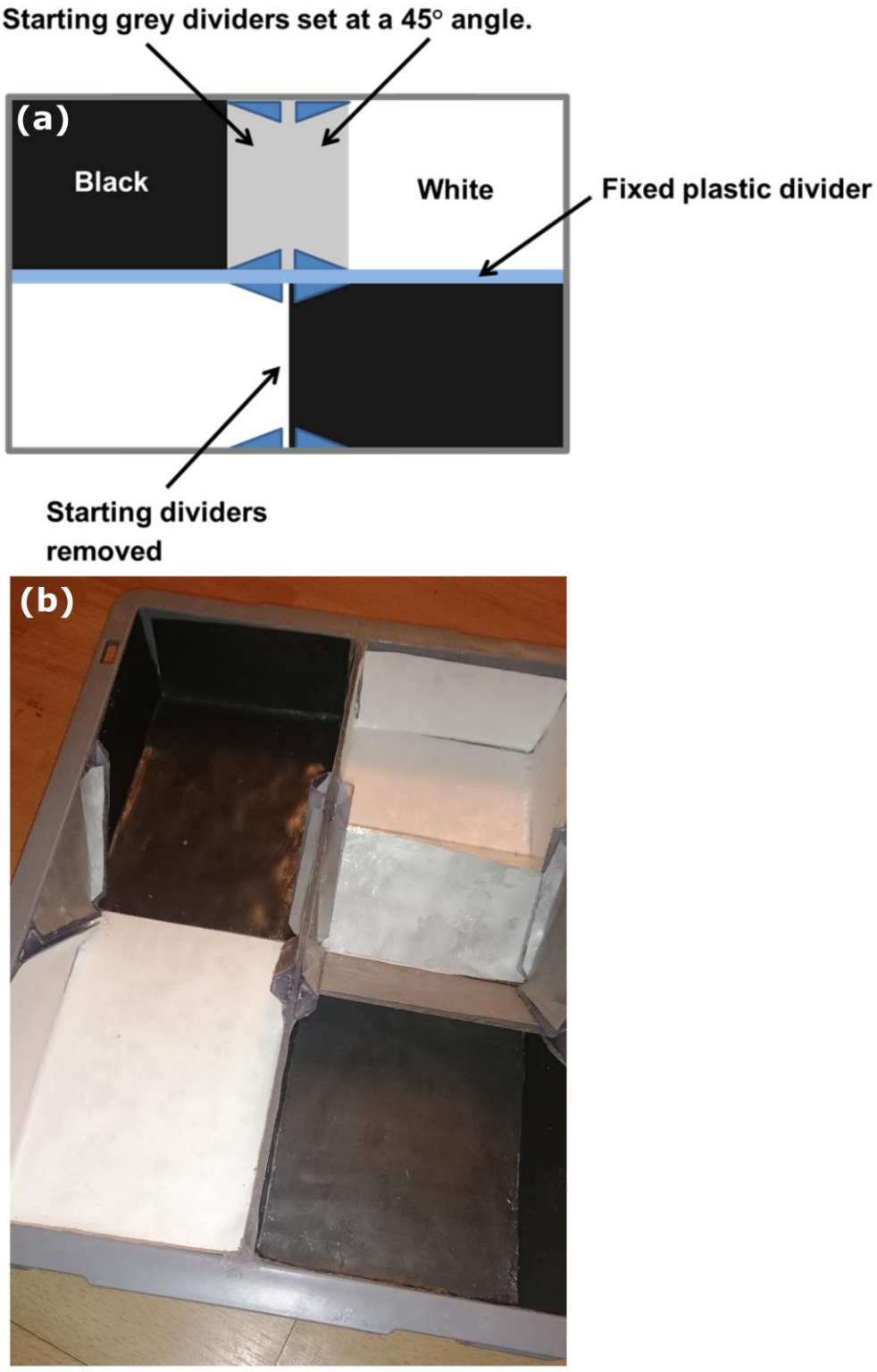

Starting grey dividers set at a 45° angle.
(a)
Black
White
Fixed plastic divider
Starting dividers removed
(b)

Figure A4

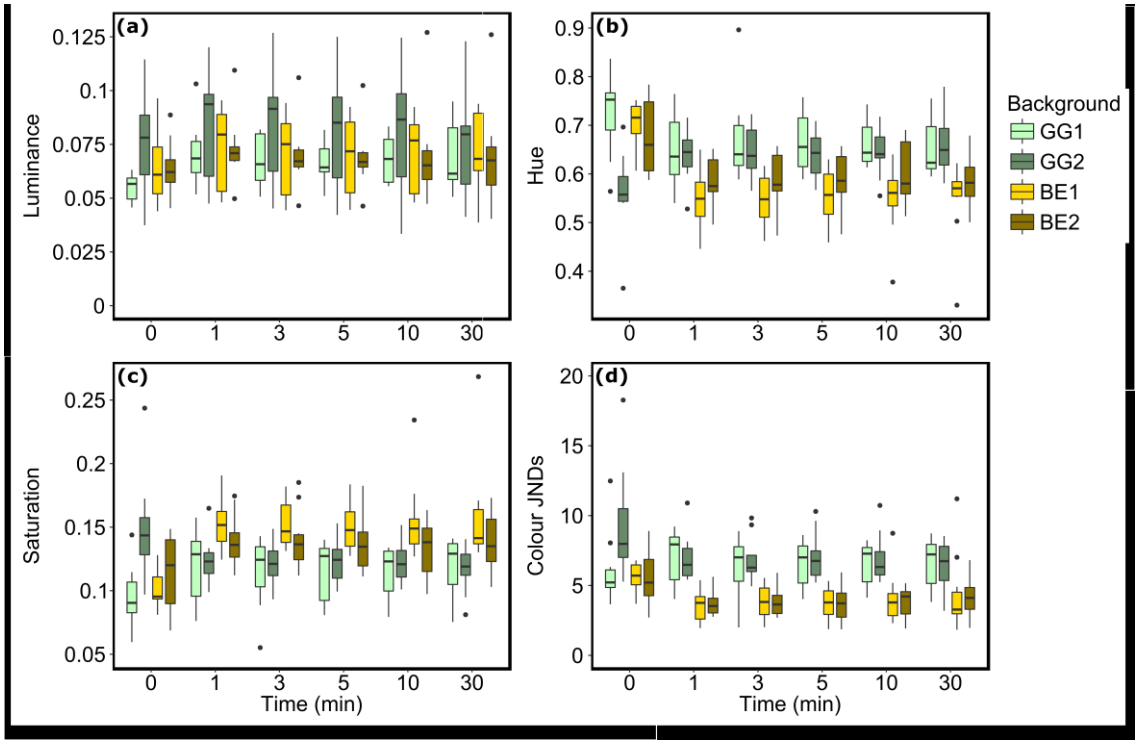


Figure A5

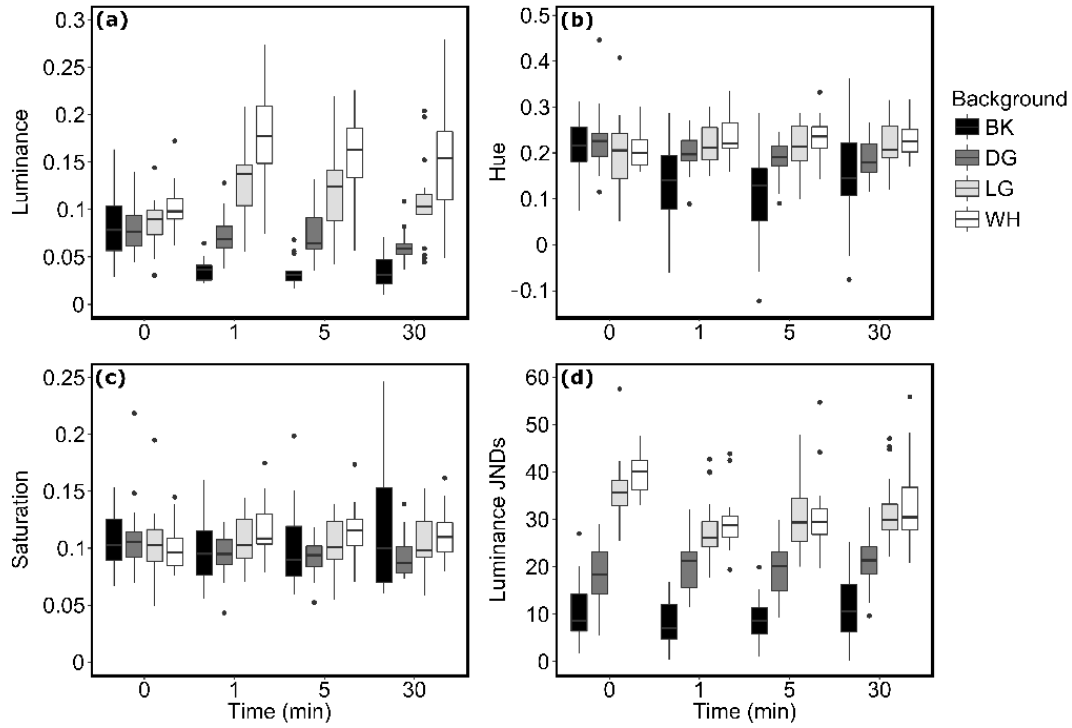


Figure A6